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Running title: Diet modifies terminal investment

Abstract

When future reproductive potential is threatened, for example following infection, the terminal investment hypothesis predicts that individuals will respond by investing preferentially in current reproduction. Terminal investment involves reallocating resources to current reproductive effort, so it is likely to be influenced by the quantity and quality of resources acquired through diet. Dietary protein specifically has been shown to impact both immunity and reproduction in a range of organisms, but its impact on terminal investment is unclear. We challenged females from ten naturally-derived fruit fly (*Drosophila melanogaster*) genotypes with the bacterial pathogen *Pseudomonas aeruginosa*. We then placed these on either a standard or isocaloric high protein diet, and measured multiple components of reproductive investment. As oogenesis requires protein, and flies increase egg production with protein intake, we hypothesized that terminal investment would be easier to observe if protein was not already limiting. Oral exposure to the pathogen triggered an increase in reproductive investment. However, while flies feeding on a high protein diet increased the number of eggs laid when exposed *to P. aeruginosa*, those fed the standard diet did not increase the number of eggs laid but increased egg-to-adult viability following infection. This suggests that the specific routes through which flies terminally invest are influenced by the protein content of the maternal diet. We discuss the importance of considering diet and natural routes of infection when measuring non-immunological defences.

Key-words: dietary protein; *Drosophila melanogaster*; fecundity compensation; non-immunological defence; oral infection; *Pseudomonas aeruginosa;* terminal investment

Introduction

Organisms have evolved an array of strategies to minimize the impact of infection on fitness, including behavioral avoidance of infection (Curtis, 2014; Vale *et al.*, 2018), and mechanisms that either mediate pathogen clearance or that minimize the damage caused by pathogen exploitation (Gupta & Vale, 2017; Soares *et al.*, 2017; Lissner & Schneider, 2018). These defense mechanisms are often costly to maintain and deploy (Moret & Schmid-Hempel, 2000; Armitage *et al.*, 2003; Bonneaud *et al.*, 2003; Duncan *et al.*, 2011; Auld *et al.*, 2013; Susi & Laine, 2015; Vale *et al.*, 2015). For example, investing in immunity may be costly if it reduces the resources available for other somatic functions, such as growth, tissue repair or reproduction (Schwenke *et al.*, 2016). The optimal resource allocation strategy will vary according to individual condition and environmental context, and a key trade-off is that between current and future reproduction (Williams, 1966; Holliday, 1989). When future reproductive potential is threatened, spreading reproductive investment over multiple breeding attempts may result in reduced fitness relative to individuals investing more in current reproduction. The terminal investment hypothesis predicts that individuals will respond to cues of impending sterility or mortality with increased investment in current reproduction (Minchella & Loverde, 1981; Clutton-Brock, 1984; Thornhill *et al.*, 1986).

Terminal investment may take the form of increased early reproductive output, early maturation, or an increase in other forms of reproductive investment such as mating effort or parental care (Duffield *et al.*, 2017). Terminal investment has been observed in diverse animal and plant taxa in response to a wide range of cues (reviewed in Duffield *et al.*, 2017), including resource availability (Kim & Donohue, 2011), injury (Morrow *et al.*, 2003) non-pathogenic immune stimulation (Bonneaud *et al.*, 2004; Jacot *et al.*, 2004) and infection by lethal (Waldman *et al.*, 2016; Gupta *et al.*, 2017a), sub-lethal (Roznik *et al.*, 2015; Gupta *et al.*, 2017a) or sterilizing (Minchella & Loverde, 1981; Chadwick & Little, 2005; Vale & Little, 2012) pathogens. Because it increases host fitness during infection without directly reducing pathogen burdens, terminal investment increases host disease tolerance, and has been

described as an adaptive, non-immunological defense against infection (Parker *et al.*, 2011; Kutzer & Armitage, 2016a).

While terminal investment may be triggered by cues of impending sterility, harm or mortality, recent discussions have highlighted how the propensity to terminally invest may depend on other external or environmental factors, where there is a dynamic terminal investment threshold (Duffield *et al.*, 2017). The main points of this hypothesis are that the extent of terminal investment may be context dependent, and that the terminal investment response may only be observed above a certain level of a given environmental factor. One such factor likely to be important for terminal investment is diet. Terminal investment reallocates resources from other somatic functions to current reproductive effort, and should therefore rely heavily on the acquisition of dietary nutrients, their transformation into energy resources, and the appropriate allocation of these resources to different life-history traits (Schwenke *et al.*, 2016). Diet is known to affect both fecundity and immunity across a wide range of species (Lee *et al.*, 2008; Maklakov *et al.*, 2008; Jensen *et al.*, 2015; Schwenke *et al.*, 2016; Rapkin *et al.*, 2018), and protein in particular is a key resource for growth, development and reproduction (Mirth *et al.*, 2019). Fruit flies (*Drosophila melanogaster*) produce more eggs on protein rich diets and these eggs are more likely to be viable (Drummond-Barbosa & Spradling, 2001; Lee *et al.*, 2008; Lihoreau *et al.*, 2016; Mirth *et al.*, 2019). Egg protein content is influenced directly by dietary protein (Kutzer & Armitage, 2016b; Mirth *et al.*, 2019) and has been shown to correlate with hatchling size (Stahlschmidt *et al.*, 2013). Egg protein content may additionally be subject to trade-offs against the immune response, evidenced by immune challenged female mosquitoes (*Anopheles gambiae*) laying eggs with lower protein content (Ahmed *et al.*, 2002). Despite these findings, and a growing body of work showing an important role of dietary protein on immune responses (Lee *et al.*, 2006, 2008; Ponton *et al.*, 2018), few studies have investigated how diet or specific nutrients influence terminal investment (Jacot *et al.*, 2004; Krams *et al.*, 2015).

In the present study we tested the effect of dietary protein on terminal investment in the fruit fly *D. melanogaster.* Previous work on systemic infection in *Drosophila* reared flies on either a standard or reduced protein diet but did not find any evidence for increased reproductive output following infection on either diet (Kutzer & Armitage, 2016b). Due to the expected trade-off between reproduction and immunity(Schwenke *et al.*, 2016), and the elevated protein requirements of oogenesis, we hypothesized that terminal investment would be easier to observe when protein was not already limiting egg production. We exposed female flies orally to the bacterial pathogen *Pseudomonas aeruginosa* to establish an enteric infection. We placed flies on a standard cornmeal-sugar-yeast Lewis diet (Lewis, 2014) or on a modified, isocaloric, high protein diet, and measured reproductive outputs to assess the role of dietary protein on the reproductive quantity and also on the number of eggs that eclosed as viable offspring.

Methods

Fly lines and rearing conditions

We used ten lines from the *Drosophila* Genetic Reference Panel (DGRP): RAL-59, RAL-75, RAL-138, RAL-373, RAL-379, RAL-380, RAL-502, RAL-738, RAL-765 and RAL-818 (Mackay *et al.*, 2012). These lines have been previously shown to vary in a number traits related to their immune, physiological and behavioural responses to pathogens (Magwire *et al.*, 2012; Bou Sleiman *et al.*, 2015; Siva-Jothy & Vale, 2019b; a). All lines were previously cleared of *Wolbachia* infection, which is known to confer protection against enteric bacterial infection by *P. aeruginosa* (Gupta *et al.*, 2017b). Stocks were kept at 25 °C under a 12:12 light:dark regime at densities of 10-20 adult flies per vial, which were allowed to lay for 24 hours before being removed. Flies laid for the experimental generations were density controlled by adding 15 female and 2 male flies to each vial for 24 hours on a standard Lewis diet without additional yeast, as larval diet is known to later influence oogenesis in adult flies (Aguila *et al*.,2012), while we were interested in the role of current protein content of diet in mediating life history responses to infection. The resulting adult offspring were lightly sedated with CO_2, and divided into two density-controlled vials for each line by placing 15 females and 2 males on standard Lewis diet for 24 hours to ensure mating had occurred prior to the experiment.

Diet treatments and experimental setup

Two diets of differing protein levels were used (Tables S1 and S2). A standard Lewis diet of roughly 14% protein was chosen, as this is frequently employed in laboratory experiments involving *Drosophila*. The second diet was a Lewis diet modified to contain approximately double the amount (~31%) protein, previously shown to induce significantly higher egg laying in *Drosophila* (Lee *et al.*, 2008; Jensen *et al.*, 2015). Protein quantity was manipulated by increasing the yeast component, while carbohydrate was reduced by decreasing the sugar to maintain an approximately isocaloric diet (Tables S1 and S2). Both diets were dyed with

Brilliant Blue FCF E133 (Sigma) to increase contrast between the eggs and the food during egg counts. The experiment used a balanced 2×2×10 fully cross-factored design, with two levels of infection status (infected and uninfected), the two diets, and ten fly lines. Ten, individually housed replicate flies from each line were subjected to each treatment, for a total of 400 flies, or 100 flies for each diet-infection status combination, all divided evenly between two blocks.

Pseudomonas aeruginosa culture and oral infection protocol

P. aeruginosa reference strain PA14 is a gram-negative bacterium known to cause mortality in a range of species, including *D. melanogaster* (Apidianakis & Rahme, 2009; De Soyza *et al.*, 2013). Bacterial cultures were grown overnight and resuspended in 5% sucrose solution to achieve an OD_{600}=25 as described previously (Siva-Jothy *et al.*, 2018). Flies were starved for 7-8 hours prior to infection by tipping into foodless vials, bunged with absorbent cotton wool moistened with distilled water to prevent dehydration. In the 24 hours preceding the infection protocol, 500µl of sugar agar (20g of agar powder and 84g of brown sugar, dissolved in 1l distilled H_2O and heated) was added to the lid of a 7ml Bijou tube (Fisher Scientific 129A). Once firm, a 20mm filter paper disc was placed on the agar, and the bijous were sealed for overnight storage at 4°C, and returned to room temperature before use. Immediately before infection, 80µl of the PA14 suspension (OD_{600}=25 as described above), or 5% sucrose for the control, was added onto the filter disc and allowed to dry for 20 minutes. The starved flies were lightly sedated with CO_2, transferred to individual bijous and kept overnight (~16 hours) at 25°C to ingest bacteria. They were then tipped onto their designated diets. Infection, and the absence of contamination of the controls was confirmed by surface sterilizing, homogenizing, and plating additional flies subject to the infection protocol alongside experimental flies.

Fecundity and survival following infection

Following infection, the flies were housed individually on either the standard Lewis diet, or the modified higher protein diet (described above), and maintained at 25°C on a 12:12 light:dark

cycle. All flies were tipped onto fresh food of the same diet every day for seven days, when their survival was recorded, and the number of eggs laid counted under a microscope. Survival was recorded for an additional 3 days after egg counts concluded. To assess egg-to-adult viability, eggs laid on days 1-3, 5 and 7 were incubated for 16 days at 25°C, and the number of eclosed offspring were counted.

Analysis

Analysis and plots were performed using R version 3.4.3 (Core Team, 2017) using the packages *lme4* (Bates *et al.*, 2015) and *survival* (Therneau, 2015). All models include the random effect of individual nested within line to account for repeated measures across individuals and lines. Daily egg production and number of eclosed offspring were analyzed via generalized linear mixed effects models (GLME). Models fitted diet, infection status, day and their interactions, alongside block as categorical fixed effects. To control for overdispersion within the data, row ID was included as a random effect in both models. Egg-to-adult viability was analysed using a binomial GLME, with the number of eggs that eclosed and the number which did not eclose bound and treated as the response variable, i.e. the proportion of eggs eclosing. Diet, infection status, day and their interactions were treated as fixed effects as well as block. To account for potential density effects, the total number of eggs present in the vial was included as a random effect. To understand any life-history changes induced solely by diet, infection and its interactions were dropped and all models were rerun on control flies only. Full R code for all analysis is available in electronic supplementary material.

Results

Life-history changes due to dietary protein in uninfected flies

Before examining the effects of dietary protein on terminal investment, we assessed its effects on reproductive output in healthy flies. Flies reared on the high protein diet produced more eggs than those on the standard diet (Figure 1), and these eggs showed higher viability (Figure 2), resulting in more eclosed adult offspring per fly each day under high protein compared to the standard diet (Figure 3; Table 1). The number of eggs laid daily increased over the course of the experiment when flies were fed the high protein diet, but this increase was not as evident under the standard protein diet (Figure 1, light blue bars; Table 1, 'Diet x Day' interaction). Diet-dependent temporal dynamics were also evident for the number of viable offspring (Figure 2; Table 1, 'Diet x Day' interaction). We found that the genetic background of flies contributed significantly to the variance in both the number of eggs laid and in egg-to-adult viability (Table 1 "line" effect; Figures S1-S3).

Increased oviposition in infected flies on high protein diet

Flies exposed orally to *Pseudomonas aeruginosa* experienced significantly higher mortality than control flies, but the rate of mortality and the microbe load within flies did not differ with diet (Figure S4). Most mortality (approximately 40%) occurred within 1-3 days following oral exposure, reaching 50% by the end of the experiment. The genetic background of the flies explained a significant proportion of variance in the number of eggs laid (Table 2 "line" effect; Figures S1). Flies exposed to *P. aeruginosa* laid significantly more eggs than those exposed to a control solution, but only when fed the high protein diet (Figure 1; Table 2, Model 1 'Diet x Infection Status'). Averaged over all days, exposed flies on the high protein diet laid 9.3 eggs per day, compared to 7.6 laid per day by control flies on the same diet.

Egg viability is increased in infected flies, regardless of diet

While increasing the number of eggs following exposure to a pathogen is a clear indication of terminal investment, more eggs will only translate into increased fitness if they are capable of developing into viable adult offspring. Infected flies on the high protein diet produced a greater number of viable offspring than those on the standard diet (Figure 3; Table 2, Model 2, 'Diet x Infection'), reflecting increased egg laying. Additionally, egg-to-adult viability was higher in infected flies than controls. Flies on the standard diet showed a larger increase in viability following infection than those on the high protein diet, peaking 2 days post-infection. Both the total number of eclosed offspring and the egg-to-adult viability differed between fly lines (Table 1 "line" effect; Figures S2-S3).

Discussion

We investigated the effect of dietary protein on terminal investment in response to infection, a form of non-immunological defense that mitigates the potential fitness losses of infection by increasing reproductive investment (Parker *et al.*, 2011; Kutzer & Armitage, 2016a). We found that oral infection by *P. aeruginosa* was sufficient to trigger a shift in reproductive investment, recapitulating similar increases in reproductive output in *D. melanogaster* following sub-lethal viral infections (Gupta *et al.*, 2017a). Given the elevated protein requirements of oogenesis (Mirth *et al.*, 2019), we hypothesized that terminal investment would be more likely to be observed when protein was not limited. We observed increased reproductive investment in both diet treatments, though the nature of these terminal investment strategies depended on the availability of dietary protein. Flies feeding on a high protein diet invested terminally in the quantity of eggs, while flies fed the standard diet showed an increase in the viability but not quantity of their eggs.

While there is a considerable amount of work showing that protein levels affect reproductive output and immunity (reviewed in Schwenke *et al.*, 2016), the role of diet on the ability to

terminally invest following exposure to pathogens has received less attention, but is likely to be driven by differences in nutrient-mediated trade-offs between immunity and reproduction. In one study, diet-restricted male mealworm beetles (*Tenebrio molitor*) were found to invest terminally in attractive sex odours at the expense of a resistant encapsulation response to a nylon implant (Krams *et al.*, 2015). In other work, reduced reproductive investment (mate calling) by male crickets (*Gryllus campestris*) injected with bacterial lipopolysaccharides was augmented by dietary supplementation (Jacot *et al*. ,2004*)*. The nature of the trade-offs experienced between immunity and reproduction may even present more complex sex-specific patterns, as shown in the tropical house cricket *Gryllodes sigillatus* where female encapsulation ability (a component of immune defence) and egg production increased with the intake of both protein and carbohydrates, whereas male encapsulation ability increased with protein intake but calling effort (a measure of investment in reproduction) increased with carbohydrate intake (Rapkin *et al.*, 2018).

A potentially important caveat to the interpretation of our results is that we did not measure the consumption, which could result in different intakes of protein and carbohydrate if feeding rate differs between standard and high protein diets. While accurate recording of consumption is an important consideration in the field of nutritional geometry, a key take home message from this work is that it is what you eat and not how much (Lee, 2015; Moatt *et al.*, 2016). Given the key role of protein, particularly in females and invertebrates (Piper *et al.*, 2011; Fanson *et al.*, 2012; Moatt *et al.*, 2016; Mirth *et al.*, 2019), we chose to interpret our results in relation to protein content of the diet. In *Drosophila*, both the quantity of protein per egg and the quantity of eggs produced are influenced by dietary protein availability (Mirth *et al.*, 2019). Female *D. melanogaster* typically weigh 800-1100µg (Jumbo-Lucioni *et al.*, 2010), and lay eggs containing approximately 10-12µg of protein each (Kutzer & Armitage, 2016b). The highest laying fly in this study produced 172 eggs over 7 days, representing about 2000µg of protein invested in egg production, or ~200 % of the fly's wet weight, which underlines the importance of dietary protein for oogenesis. In the current experiment, flies on the high protein

diet produced more eggs than flies on the standard diet. The lack of terminal investment in the number of eggs on the standard diet may therefore be a result of the necessary protein being unavailable. It is therefore plausible that other studies where terminal investment has not been observed were a result of insufficient protein being available to terminally invest in increased reproduction.

Investing in increased egg production is one way organisms can improve their number of surviving offspring, but another is to ensure that the offspring produced are viable. We found that the greatest increase in egg-to-adult viability following infection was observed in eggs laid by flies on the standard diet. Some caution is warranted when interpreting these results, as we cannot completely separate the effects of increased egg density between diets, which would especially impact the viability to flies reared in the high protein diet. However, such effects would not explain why we see an increase in viability specifically in the infected treatment. Another potentially confounding factor is that eggs laid by infected flies could have been exposed to maternally shed pathogens, which could affect egg viability. We tested for this possibility by plating a sample of the eclosed flies, but we did not detect any evidence of maternal contamination, and it would not be clear how such exposure pathogens would increase viability. Instead, there is precedent for an effect of protein on egg-to-adult viability from showing that flies raised on a poor diet produce heavier eggs, and produce offspring that themselves are more resistant to poor nutrition than those of flies raised on a standard diet (Vijendravarma et al., 2010).This suggests that flies may be subject to a protein allocation trade-off between per-egg protein allocation, and number of eggs produced, and that payoffs of this trade-off vary according to the quality of food available. In a situation of limited protein availability, it may be better to invest what little protein is available in a smaller number of eggs to improve offspring viability.

Compared to previous work on terminal investment, particularly in insect systems, a potentially important aspect of this study was the infection method. We chose to establish a gut infection

because we were investigating an evolved adaptive response to infection, and oral infection by *Pseudomonas* is believed to be more common in the wild than infection via septic route employed in many studies. Other work has shown that the evolutionary response of *D. melanogaster* to *Pseudomonas* infection is specific to the route of infection (Martins *et al.*, 2013), and that antibacterial protection by *Wolbachia* occurs during oral but not systemic infections (Gupta *et al.*, 2017b). These results suggest that selection to cope with oral *Pseudomonas* infection has been stronger, which may explain why previous works which often employed systemic infections have not detected a similar terminal investment response (Kutzer & Armitage, 2016b).

The precise mechanisms by which changes in diet affect reproductive traits following infection are difficult to disentangle. Dietary protein provides both the raw material for egg production, as well as influencing complex signalling pathways which determine investment in egg production (Mirth *et al.*, 2019). Our results showed that flies on the standard diet could produce eggs with higher viability but did not invest in doing so in the absence of infection. This suggests that raw materials were available to produce more viable eggs, but signalling pathways controlling investment in egg viability were influenced by limited protein availability to reduce this investment. Recent research has highlighted the roles played by juvenile hormone and ecdysone levels as well as insulin signalling in regulating egg production in response to nutritional states (Mirth *et al.*, 2019). Additionally, bacterial derived peptidoglycans have been shown to activate NF-kB signalling pathways in octopaminergic neurons, resulting in changes in egg laying (Kurz *et al.*, 2017). Interactions between these pathways signalling nutritional and infection status may therefore underlie protein-mediated changes in terminal investment. Future work should investigate these interactions and attempt to characterise their potential as a mechanism by which organisms can pursue optimal strategies under differing nutrient availabilities.

In summary, we find that dietary protein can mediate the terminal investment strategy of flies following infection. This result places our current understanding of non-immunological defence

from infection in an important ecological context, as environments where protein availability is variable may select for multiple resource-dependent strategies for limiting the impact of infection. Further research into the wider consequences on the population ecology of host species during infection, and the underlying physiological mechanisms of these responses is now needed. Combined, this will result in a clearer understanding of the broader ecological and evolutionary implications of fluctuating resource availability in natural populations.

Data accessibility

Data is available in supplementary electronic material. All data and code for analysis will be deposited in Dryad upon publication.

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Author contributions

ALH and PFV conceived and designed the study. ALH carried out experimental work and collected the data. ALH, JPM and PFV analysed the data. ALH drafted the manuscript. ALH, JPM and PFV wrote the final version of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Figures and Tables

Table 1: Summarized models of control non-infected flies only.

Term	**Model A:** Eggs counts		**Model B:** Viable Offspring		**Model C:** Egg-to-Adult Viability	
	χ^2	**P=**	χ^2	**P=**	χ^2	**P=**
Diet	6.15	0.013	53.04	<0.0001	63.67	<0.0001
Day	276.98	<0.0001	105.86	<0.0001	10.74	0.030
Line	43.73	<0.0001	0.29	0.59	394.64	<0.0001
No. of Eggs Laid	-	-	-	-	352.28	<0.0001
Block	8.21	0.0042	1.53	0.22	3.57	0.59
Diet × Day	40.24	<0.0001	15.14	0.0044	4.45	0.35

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Table 2: Summarized models for infected and control flies.

Term	**Model 1:** Eggs counts		**Model 2:** Viable Offspring		**Model 3:** Egg-to-Adult Viability	
	χ^2	**P=**	χ^2	**P=**	χ^2	**P=**
Diet	17.26	<0.0001	69.87	<0.0001	73.55	<0.0001
Infection	0.0223	0.88	30.41	<0.0001	40	<0.0001
Day	307.14	<0.0001	123.6	<0.0001	61.69	<0.0001
Line	71.7	<0.0001	21.62	<0.0001	98.92	<0.0001
No. of Eggs Laid	-	-	-	-	29.79	<0.0001
Block	12.35	<0.001	1.71	0.19	13.06	<0.001
Diet × Infection	4.45	0.035	6.54	0.011	24.99	<0.0001
Diet × Day	76.37	<0.0001	43.22	<0.0001	65.52	<0.0001
Infection × Day	75.23	<0.0001	37.12	<0.0001	23.12	<0.001
Diet x Infection × Day	6.88	0.33	1.39	0.85	22.37	<0.001

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Figure Legends

Figure 1 - Egg Production. Mean ± SEM number of eggs laid per fly by control flies (light blue) and infected flies (dark blue) on the first seven days following infection on the standard Lewis diet (top) and the modified high protein diet (bottom).

Figure 2 – Total Viable Offspring. Mean ± SEM number of eclosed offspring per fly by control flies (light blue) and infected flies (dark blue) over seven days following infection on the standard Lewis diet (top) and the modified high protein diet (bottom).

Figure 3 - Egg-Adult Viability. Proportion of eggs which eclosed laid by control flies (light blue) and infected flies (dark blue) over seven days following infection on the standard Lewis diet (top) and the modified high protein diet (bottom).